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Phenotypical heterogeneity in responder (R) and non-responder (NR) male ApoE*3Leiden.CETP mice

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Running title: Phenotypical characterization of ApoE*Leiden.CETP mice

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Abbreviations:

MetS: Metabolic syndrome

R: responder

NR: non-responder

HFHC: high fat high cholesterol diet

Keywords: metabolic syndrome, heterogeneity and phenotype, liver functionality, inflammation

Abstract

The metabolic syndrome (MetS) is a major health issue worldwide and is associated with obesity, insulin resistance, and hypercholesterolemia. Several animal models were used to describe the MetS, however many of them do not mimic well the MetS pathophysiology in humans. The ApoE*3Leiden.CETP mouse model overcomes part of this limitation, since they have a humanised lipoprotein metabolism and a heterogeneous response to MetS, similarly to humans.

The reported heterogeneity among them and their common classification into responder (R) and non-responder mice (NR); R mice show increased body weight, cholesterol and triglycerides levels, while NR mice do not show this expected phenotype when fed a Western type diet. To better define the differences between R and NR mice, we focused on feeding behavior, body weight gain, glucose tolerance, and lipid parameters, and on an extensive pathological examination along with liver histology analysis.

Our data confirmed that R mice resemble the pathological features of the human MetS: obesity, dysplipidemia, glucose intolerance; NR mice do not develop the full dysmetabolic phenotype because of a severe inflammatory hepatic condition, which may heavily affect liver function.

We conclude that R and NR mice are metabolically different and that NR mice have indications of severely impaired liver function. Hence, it is critical to identify and separate the

53 respective mice to decrease data heterogeneity. Clinical chemistry and histological analysis
54 should be used to confirm retrospectively the animals' classification. Moreover, we point out
55 that NR mice may not be an appropriate control for studies involving ApoE*3Leiden.CETP R
56 mice.
57
58 New and Noteworthy
59 Compared to some other animal models, ApoE*3Leiden.CETP mice are better models to
60 describe the metabolic syndrome. However, there is phenotypic heterogeneity between
61 "responder" and "non-responder" mice, the latter showing some evidence of hepatic
62 pathology. A full phenotypic characterization and eventually *post mortem* analysis of the liver
63 is warranted.
64

Introduction

Obesity in humans (defined as Body Mass Index, BMI>30 kg/m^2) is one of the major health problems worldwide and is expected to increase even more in the next decades (10). Although genetics factors are known to be involved in the pathogenesis of obesity (39, 64), its onset in the majority of people is due to excessive energy intake combined with a lack of physical activity. Obesity is often associated with other comorbidities such as cardiovascular diseases (CAD) (40), type 2 diabetes mellitus (T2DM) (57), hypertriglyceridemia (2), and non-alcoholic fatty liver disease (NAFLD) (3). Together these conditions form the metabolic syndrome (MetS) (24). Since obesity may induce insulin resistance or changes in lipoprotein metabolism, it may be considered the major driving force behind the MetS (12). While not all the underlying pathophysiological mechanisms are completely understood, the number of MetS patients increases (9). Thus, understanding and deciphering these mechanisms will hopefully provide new means to counteract the MetS epidemic.

Several animal models, such as the diet-induced obese (DIO) mouse (58), the leptin-receptor deficient *db/db* mouse (22) or the Zucker rat (65), have been used in the past decades to try to understand the consequences of a Western type diet on metabolism; however they typically do not develop all aspects of the MetS; in particular, the underlying genetic defects in the *db/db* mouse and the Zucker rat do not replicate the typical human pathophysiology. Moreover, there are animal models in which features of the MetS (19) develop spontaneously on chow diet as for example in the DahlS.Z-*Lepr*$^+$/*Lepr*$^+$ rat (13), the Nile grass rat (33) or ZDSD rat (38). This does not occur in human patients, who rarely show sign of the MetS when fed a balanced low fat diet; usually, only when eating a Western type diet, the typical MetS symptoms develop. Taking this into account, in our studies, we used the double transgenic mouse model ApoE*3Leiden.human Cholesterol ester transfer protein (ApoE3L.CETP). The ApoE3Leiden mutation (14, 60) and the introduction of human CETP gene (60) make these mice' lipoprotein metabolism more similar to humans, thus making them one of the best animal models available to assess lipid metabolism in response to a

Western type diet (e.g. a 60% high fat supplemented with 0.25% cholesterol (HFHC) diet) (14, 60). Hence, these mice show a humanized lipoprotein metabolism, develop atherosclerosis when fed HFHC diet, and display a human-like response when treated with statins (51, 53). Compared to wildtype mice, they have higher concentrations of very low density lipoprotein (VLDL) and low density lipoprotein cholesterol (LDL-C), and relatively low concentrations of high density lipoprotein cholesterol (HDL-C) when fed the HFHC diet. The presence of the ApoE3Leiden transgene hampers the uptake of VLDL remnants by the liver leading to increased VLDL/LDL-C levels in plasma, similar to humans (60). Further, the introduction of the human glycoprotein CETP is responsible for the transport of cholesterol ester from HDL to apolipoprotein-B (apo-B) containing lipoproteins in exchange of triglycerides, leading to decreased HDL-C (60).

The major aim of this study was to characterize the effect of long-term access to the HFHC diet in the ApoE3L.CETP mice on feeding behavior, and glucose and lipid metabolism. Interestingly, responses of ApoE3L.CETP mice to the HFHC diet are highly variable and therefore an attempt was made to clarify the causes of the observed heterogeneity inherent to this model. Our study highlighted in detail the presence of two different phenotypes within the same genetic background in ApoE3L.CETP mice: responder (R) mice and non-responder (NR) mice.

We characterized the morphological, metabolic and functional aspects of these two phenotypes through an extensive metabolic and histologic liver analysis of this mouse model. Our data indicate that only R ApoE3L.CETP mice recapitulate adequately the salient pathological features of human MetS. NR mice develop instead a prominent inflammatory hepatic condition that has detrimental effects on liver function, and may prevent the development of the expected “MetS phenotype”. Hence, the results of this study indicate that the high heterogeneity observed in this model might affect study outcomes, without an accurate distinction being made between R and NR animals.

Material and Methods

Animals and husbandry

Male ApoE3L.CETP mice (TNO, Innovation for Life, The Hague, Netherlands) (37, 60) aged 7-19 weeks were used in all experiments. Prior to be delivered to our facility, 6-7 weeks old mice kept on a standard chow diet were tested by TNO for plasma total cholesterol and triglycerides levels; mice with triglycerides levels lower than 2 mmol/L (i.e. 177 mg/dL) were considered as NR mice, while those with triglycerides levels above this arbitrary threshold were classified as R mice. In the cohorts used in our experiments, particularly, NR mice had total cholesterol levels between 1.4 – 2.2 mmol/L (i.e. 54.1 - 84.9 mg/dL), and triglycerides between 0.6 - 1.9 mmol/L (i.e. 53.1 - 168.1 mg/dL), while R mice total cholesterol was higher than 1.9 mmol/L (i.e. 73.4 mg/dL) and triglycerides higher than 2.1 mmol/L (i.e. 185.8 mg/dL) at 6-7 weeks of age. For consistency, we will report henceforth our study data only in mg/dL units, and refer to both mmol/L and mg/dL only for data collected at TNO.

Two different cohorts of R mice and NR mice were ordered from TNO. Upon arrival at our facility, R ApoE3L.CETP mice and NR ApoE3L.CETP mice separated based on TNO plasma results, were group housed as they were shipped (2-5 mice per cage). Mice were housed at a temperature of 21°C with an artificial 12h light-dark cycle (light on 2:00 am, light off 2:00 pm). Individual mice had to be isolated due to fighting reasons, whilst a group of mice (cohort 2, group 3 in Table 1) was single-housed for indirect telemetry experiment. R mice and NR mice used in the different experiments were in the same range of age.

They were acclimated to the new housing and environmental conditions for 2 weeks prior to the start of the experiments. During the acclimation period and for the following 2 weeks, mice were fed a chow diet (22.4% protein, 12.3% fat and 65.4% carbohydrate, #3436, Kliba Nafag, Kaiseraugst, Switzerland) after which they were switched to a high fat high cholesterol diet containing 60% kcal fat and 0.25% cholesterol (D14010701, HFHC diet, Research Diets, New Brunswick NJ, USA) until the end of the experiments. This was a custom diet modified from the original 60% kcal diet containing 25% protein, 36% fat and

25% carbohydrate. More detail on the experimental design is given in Table 1. The Cantonal Veterinary Office of Zurich approved all the animal experiments (licence 122/2014).

Animal health

Mice imported from TNO were negative for endo- and ectoparasites, viruses and bacteria, as indicated by the latest FELASA-compliant health reports (results not shown). Sentinel mice at our facility were tested quarterly and resulted negative for major etiologic agents. Additional testing was conducted to confirm the specific pathogen free health status for *Helicobacter spp*. using qPCR on pooled frozen liver samples obtained from R mice and NR mice used in this study (n=3). The qPCR analysis was conducted in a diagnostic laboratory (Idexx Bioresearch, Ludwigsburg, Germany) using *Helicobacter* genus and *Helicobacter* species-specific PCR primers.

Body weight and body composition

Body weight was measured weekly from arrival at the facility until the end of the experiments. After sacrifice, 7 mice from each group were subjected to CT scanning (LaTheta LCT-100A scanner, Hitachi-Aloka Medical Ltd., Tokyo, Japan) for body composition, and the proportion between lean and fat mass (subcutaneous and intra-abdominal adipose tissue) in the region between lumbar vertebrae L1-L5 was analyzed using LaTheta software (version 2.10, Hitachi-Aloka Medical Ltd.) (15, 56).

Feeding behavior

Individual food consumption was investigated for an entire week in single-housed mice. 24-hour cumulative food intake was manually assessed then a proper feeding behavior study was performed.

After 6 weeks of HFHC diet feeding, R mice (n=8) and NR mice (n=8) were single-housed in BIODAQ cages (Research Diets, New Brunswick, NJ, USA) equipped with external food hoppers to measure food intake continuously. Meal size and meal pattern over 24h were

then determined. Meal pattern data collected from each mouse were grouped into meals by clustering independent feeding bouts. A meal was defined as a minimum inter-meal interval (IMI) of 600 sec and minimum size of 0.02 g (48). Mice were not disturbed during these measurements.

The same mice were then transferred to an open-circuit indirect calorimetric system (TSE Phenomaster, TSE Systems GmbH, Bad Homburg, Germany) to monitor metabolic rate and respiratory gas exchange. Two weeks of adaptation were given prior to measurements for each test.

Glucose metabolism: oral glucose tolerance test and insulin sensitivity test

Oral glucose tolerance (OGTT) and insulin sensitivity (IST) tests were carried out in separate groups of R mice and NR mice to avoid stress and excessive blood sampling.

OGTTs were performed in R mice and NR mice on chow diet and after 1, 2 and 3 months of HFHC diet feeding (n=28 each time-point per group). Mice were fasted for 6h prior to the test and were briefly anesthetized with isoflurane (3% isoflurane in the induction chamber, 0.8L/min) to collect blood in microvette EDTA microtubes (Sarstedt, Nümbrecht, Germany) by sublingual vein puncture to measure basal glucose and insulin levels. At T0, a baseline blood sample was collected and a bolus of glucose (2g/kg) was given by oral gavage, after which blood was sampled by tongue bleeding at time 15 and 30 min, and by tail bleeding at time 45, 60, 90 and 120 min for glucose measurements (Breeze 2, Bayer Glucose Meter, Basel, Switzerland). The area under the curve (AUC) was calculated above the lowest glucose value of each mouse during the OGTT. Blood was kept on ice and centrifuged at 4°C for 5 min at 11000 rpm. EDTA plasma was separated and stored at -80°C until assayed. Insulin levels at time-points 0, 15 and 30 min were measured with a specific Mouse Insulin ELISA (Mercodia AB, Uppsala, Sweden) (Data not shown).

ISTs were performed using different cohorts of R mice (n=19) and NR mice (n=15), which were fasted for 4h and bled at the same time-points (see above). After baseline blood sample measurements, insulin Humalog (0.5U/kg, 100U.I./mL, Lilly, Geneva, Switzerland)

was injected intraperitoneally and blood glucose levels were measured at 15, 30, 45, 60, 90 and 120 min after injection in blood samples collected by tail bleeding. Mice that became hypoglycemic (i.e. reached glucose levels of 2.5 mmol/L or less) were immediately given a bolus of glucose (2g/kg) and were excluded from the test for all subsequent time-points. In our analysis particularly, 9/27 R mice and 8/23 NR mice were excluded. The AUC was calculated above the lowest level of glucose of each mouse during IST test.

Cytokines and transaminase measurements

Cytokines were measured in plasma isolated from terminal blood samples with V-Plex Cytokine kit (Meso Scale Discovery, Gaithersburg, MD, USA) following the manufacturer's protocol.

Plasma alanine transaminase (ALT) was measured via UniCel® DxC 800 Synchron® Clinical System in plasma of R mice and NR mice at the time of sacrifice, according to manufacturer's instructions.

Liver and pancreas histology

All animals were deeply anesthetized by isoflurane and sacrificed via opening of the thoracic cavity and exsanguination by cardiac puncture. A complete necropsy, including a thorough external and internal gross examination, was performed on each mouse. Mice were sacrificed at different time-points after 2, 3 and 4 months of HFHC diet feeding. Organs of interest (liver, kidneys, heart, pancreas, epididymal white adipose tissue (WAT), periaortic brown adipose tissue (PBAT), and aorta) were removed, weighed (liver) and appropriately sized samples were fixed in 10% neutral-buffered formalin (Formafix, Hittnau, Switzerland) or snap frozen. After fixation, all collected organs and tissues were trimmed according to guidelines (43), dehydrated through graded alcohols and routinely paraffin wax embedded. Consecutive sections (3–5 µm) were prepared, mounted on glass slides and routinely stained with hematoxylin eosin (HE) or subjected to immunohistochemical staining. Microscopic findings in the HE-stained slides were classified with standard pathological

nomenclature and severities of findings were graded on a scale of 0 to 4 (no finding present (0), minimal (1), mild (2), moderate (3), or severe (4)). Grades of severity for microscopic findings were subjective; minimal was the least extent discernible and severe was the greatest extent possible (47). Lesions that were not graded were marked as present. Nomenclature of the microscopic observations in the liver was consistent with INHAND published guides (50). A silver stain was performed on the formalin-fixed paraffin-embedded liver sections of selected mice according to routine procedures to exclude the presence of argyrophilic bacteria.

Immunohistochemistry for the CD45 (Clone I3/2.3, Abcam ab25386, dilution 1:2000) was employed on the liver and kidneys sections of selected mice (R mice=8, NR mice=8) for the quantitative assessment of the inflammatory infiltrate. Slides were scanned in bright field at ×40 magnification using a digital slide scanner (NanoZoomer-XR C12000, Hamamatsu Photonics K.K., Japan) and the positive reaction was quantified on forty 500 μm^2-sized regions of interest randomly selected throughout the parenchyma using the Visiopharm Integrator System (VIS, version 4.5.1.324, Visiopharm, Hørsholm, Denmark). Briefly, a classification based on a threshold of pixel values allowed recognition of the positive (brown) CD45 signal and the results were expressed as area of positive-stained cells(μm^2)/mm^2 of tissue. In addition, antibodies against CD3 (Clone SP7, Bioscience M3074, dilution 1:900), CD45R/B220 (clone RA3-682, BD Pharmigen, dilution 1:800) and F4/80 (clone SP115, Invitrogen MA5-16363 dilution 1:150) were used for the characterization of T cells, B cells and macrophages, respectively. A pan cytokeratin antibody (clone PCK-26, Novus Biological, dilution 1:500) was used for the identification of biliary duct epithelial cells.

Oil red O (ORO) staining was performed on snap-frozen cryomold-embedded livers. Sections were cut at 6 µm, mounted on glass slides and allowed to dry at room temperature. They were then rinsed briefly in 50% ethanol, incubated in a 60% aqueous ORO solution (375.0 mg Oil Red O (Merck KGaA, Darmstadt, Germany) in isopropanol) at room temperature for 20 min, rinsed again in 50% ethanol and counterstained with hematoxylin for 2 min. ORO stained slides were scanned and then analyzed as previously described. For

quantification, a threshold classification was set up to allow recognition of positive (red) hepatocellular cytoplasm and negative hepatic parenchyma. The results were expressed as positive area versus total area in the whole liver section. Data are presented as average (± SEM) percentage of lipid-containing parenchyma in the group.

Triglycerides and non-esterified fatty acids in livers

Approx. 50 mg of snap frozen liver were homogenized with 1 mL 2:1 chloroform:methanol (v/v) solution to measure triglyceride content. The homogenate was centrifuged for 5 min at 2500 rpm and supernatant was collected and diluted with 0.9% NaCl. Samples were centrifuged for 5 min 2000 rpm, then resuspended in a 1:1 methanol:H_2O (v/v) solution. The upper phase was then discarded, samples were let to dry and resuspended in Dimethyl sulfoxide (DMSO). Triglycerides content was then measured with the Cobas Mira Roche-Autoanalyzer (F. Hoffmann-La Roche Ltd., Basel, Switzerland), according to the manufacturer's instructions. Non-esterified fatty acids (NEFA) were measured on approx. 10 mg frozen liver following manufacturers' procedure (Merck KGaA, Darmstadt, Germany).

Pancreatic islets isolation and glucose-stimulated insulin secretion

At time of sacrifice, whole pancreas was digested and pancreatic islets were isolated using the adapted islets isolation procedure from Nordmann et al (34) from all cohort of mice, except for mice (R mice=8, NR mice=8) in cohort 2, group 3 (Table 1). Subsequently, isolated islets were let recover for 2h in a cell culture incubator (37°C, 20%O_2 and 5%CO_2) and 10 islets/well were gently picked and plated into a 24 well ECM plate (Novamed, Jerusalem, Israel). Media was changed on day 2 and on day 4 when the glucose-stimulated insulin secretion (GSIS) test was performed following a procedure adapted from Rütti (44). Krebs-Ringer phosphate solution supplemented with 0.1% BSA and 1M Hepes was prepared (KRH). Islets were pre-incubated for 30 min in KRH supplemented with 2.8 mM glucose at 37°C to let islets adapt to KRH solution before collection of the supernatant to measure insulin secretion. To measure basal insulin release, islets were incubated for 1h

with fresh KRH + 2.8 mM glucose followed by 1h with KRH + 13.7 mM glucose to measure stimulated insulin release. After each incubation, supernatant was collected, immediately frozen and later used to measure secreted insulin. To quantify insulin content after the two incubations, islets were lysed with 0.18M HCl in 70% EtOH at 4°C overnight. Insulin was measured with the Mouse Insulin ELISA (Mercodia AB, Uppsala, Sweden) and secreted insulin was calculated as percentage of total insulin content.

Lipid parameters

Plasma total cholesterol and triglycerides were measured at TNO when mice were 6-7 weeks old to discriminate between R mice and NR mice. For consistency, we converted the unit for total cholesterol and triglyceride received from the TNO facility from mmol/L to mg/dL because all the measurements performed in our institute used this unit system. For our own measurements, an aliquot of blood collected from mice undergoing the IST was collected in Microvette Lithium-heparin vacutainers (Sarstedt, Nümbrecht, Germany) and used to analyze lipid parameters. An additional blood sample was collected at sacrifice by cardiac puncture. Blood was immediately centrifuged at 11000 rpm at 4°C for 5 min and heparin plasma was aliquoted and stored at -80°C until assayed. Triglycerides, HDL-C and total cholesterol levels were analysed via UniCel® DxC 800 Synchron® Clinical System according to manufacturer's instructions, measured in mg/dL. Non-esterified fatty acids (HR Series NEFA –HR, Wako, Virginia, USA) and beta-hydroxybutyrate (Randox D-3-Hydroxybutyrate, Randoy Laboratories Ltd., Crumlin, County Antrim, United Kingdom) were analysed in blood samples collected at sacrifice using a Cobas Mira Roche-Autoanalyzer (F. Hoffmann-La Roche Ltd., Basel, Switzerland), according to the manufacturer's instructions. CETP activity was measured in plasma collected at sacrifice with CETP activity assay (CETP activity assay kit, MAK106, Merck KGaA, Darmstadt, Germany) according to manufacturer's instructions.

315 ***Bile acids profile in plasma and feces***

316 Samples were analyzed by the Department of Laboratory Medicine, University of Groningen,
317 using 25 µL of plasma and 50 mg of feces. For plasma samples, to each sample, 250 µL
318 internal standard solution (0.1 µM D4-CA and DA-CDCA, 0.2 µM DA-TCA, DA-TCDCA, DA-
319 GCA, D6-TDCA, D4-TUDCA, D4-Tβ-MCA and D4-GCDCA) was added and vortexed.
320 Samples were then centrifuged at 11000 rpm and the supernatant transferred into a clean
321 glass tube. The liquid phase was then evaporated under nitrogen at 40°C. Before
322 measuring, samples were reconstituted in 200 µL 1:1 methanol:H_2O (v/v), vortexed for 60
323 sec and centrifuged for 3 min at 4000 rpm. The supernatant was transferred into a 0.2 µm
324 spin-filter and centrifuged at 4200 rpm for 10 min. After filtering, the samples were analyzed
325 (10 µL injection volume) by Nexera X2 Ultra High Performance Liquid Chromatography
326 system (SHIMADZU, Kyoto, Japan), coupled to a SCIEX QTRAP 4500 MD triple quadrupole
327 mass spectrometer (SCIEX, Framingham, MA, USA) (UHPLC-MS/MS) (37).
328 For feces samples, bile acid composition was determined by capillary gas chromatography
329 on a Hewlett-Packard gas chromatograph (HP 6890) equipped with a FID and a CP Sil 19
330 capillary column; length 25 m, internal diameter 250 µm and a film thickness of 0.2 µm
331 (Chrompack BV, Middelburg, The Netherlands). Bile acids were methylated with a mixture of
332 methanol and acetyl chloride and trimethylsilylated with a mixture of piridyne, N,O-Bis
333 (trimethylsilyl) trifluoroacetamide and trimethylchlorosilane. Neutral sterols were
334 trimethylsilylated with a mixture of piridyne, N,O-Bis (trimethylsilyl) trifluoroacetamide and
335 trimethylchlorosilane and measured on the same gas chromatograph equipped with the
336 same column.
337

338 ***Data Analysis and Statistics***

339 All data are expressed as mean ± standard error of the mean. Student's T-test, 2 way-
340 ANOVA followed by Tukey or Sidak post-hoc tests were used for comparison among the

groups (NR mice vs R mice), as appropriate. For all statistical analysis, a p-value lower than 0.05 was considered significant. Data were analyzed using Prism GraphPad 8.0.

RESULTS

Body weight, food intake, indirect calorimetry and body composition in R and NR mice

Upon arrival in our facility on chow diet, R mice and NR mice did not show any differences in body weight. R mice gained significantly more body weight (initial body weight 31.2 ± 0.6 g and final body weight 49.7 ± 1.1 g) compared to NR mice (initial body weight 29.0 ± 0.4 g and final body weight 39.0 ± 0.9 g) after 16 weeks of HFHC diet feeding (Fig 1A). The difference in body weight was significant from the 2nd week of HFHC diet feeding.

Body composition analysis showed no significant difference in lean mass (Fig 1F) and total fat mass between R mice and NR mice; nevertheless, NR mice had a tendency for decreased subcutaneous and significantly less intra-abdominal fat compared to R mice (Fig 1G).

Ad libitum food intake over 24h (Fig 1B), number of meals per day (Fig 1C) and parameters of energy metabolism (respiratory exchange ratio (Fig 1D) and energy expenditure (Fig 1E) revealed no difference between the two groups.

Glucose and insulin tolerance in R mice and NR mice

Oral glucose tolerance tests (OGTT) were performed at several time-points in mice on chow diet and after 1, 2 and 3 months of HFHC feeding diet, respectively (Fig 2A-D). Already when chow fed, R mice and NR mice displayed differences in their glucose metabolism (Fig 2A). Fasting (baseline) glucose levels were higher in R mice (10.4 ± 0.3 mmol/L) compared to NR mice (8.5 ± 0.3 mmol/L) and, with the exception of the 15 min time-point, this trend continued during the entire OGTT (Fig 2A). Although this difference at specific time-points, both groups were able to reach baseline values after 120 min of test, indicating similar glucose tolerance; even though AUC on chow diet of R mice was increased by 20% as compared to NR mice (Fig 2I), the difference did not reach significance.

The difference between R mice and NR mice became more pronounced when mice were HFHC diet fed. Notably, after 1 month of HFHC diet feeding, R mice became glucose intolerant with higher glucose levels as compared to NR mice during the entire OGTT (Fig 2B). This effect was exacerbated after 2 and 3 months of HFHC diet feeding (Fig 2C-D). After 3 months of HFHC diet, NR mice also slightly increased their glucose levels probably due to aging and the consequences of HFHC diet feeding (35). This was also reflected in the higher AUC (Fig 2I) compared to earlier time-points.

Insulin sensitivity tests (IST) were performed in different groups of mice at the same time-point as the OGTT (Fig 2E-H). R mice, as previously observed during the OGTT, displayed higher glucose level at times 0 and 15 compared to NR mice. Already on chow diet (Fig 2E), and after 1 month of HFHC diet feeding (Fig 2F), NR mice showed a better insulin sensitivity response compared to R mice (indicated by the higher AUC in NR mice; Fig 2J). Over time, the differences between the two groups decreased probably due to aging effect (Fig 2G-H), without reaching significance in the AUC (Fig 2J). Thus, even though R mice display higher glucose values during the IST, which indicates reduced glucose tolerance, on HFHC diet, based on the AUC during the IST, their overall insulin sensitivity was similar to NR mice (Fig 2J).

Histopathology of selected organs ~~morphology~~ and lipid profile in the liver of R mice and NR mice

The *post mortem* evaluation revealed clear differences in the liver of R and NR mice. There was no gross abnormality detected in livers of R mice euthanized at approximately 14-16 weeks of age and kept on the chow diet, whilst livers of NR mice under the same experimental conditions appeared firmer than normal and exhibited a rough granular surface with multiple ill-demarcated indentations (not shown). Livers of 20-34 weeks old R mice fed the HFHC diet for 2, 3 or 4 months, respectively, were mildly enlarged, with rounded borders, and exhibited a diffuse yellow-brown discoloration and a homogeneously smooth surface (Fig 3A). Age-matched NR mice receiving the HFHC diet presented small, firm

397 livers, with an extremely irregular granular surface, along with rare (3/30 NR mice) multifocal
398 nodular masses, 0.1-0.4 cm in diameter (Fig 3B).
399 In line with these macroscopic observations, there was a statistically significant decrease in
400 mean absolute liver weights in 20-34 weeks old, HFHC diet fed NR mice when compared to
401 R mice under the same experimental conditions (1.8 ± 0.2 vs 2.9 ± 0.3 g; $p < 0.01$) (Fig 3I).
402 The results of the histopathological analysis are presented in Table 2. Apart from the
403 presence of variably sized, predominantly periportal cytoplasmic eosinophilic inclusions (up
404 to 20 µm in diameter) within the hepatocytes, indicative of mutant Apo-E lipoprotein
405 accumulation (30), no histological abnormality was observed in the liver of R mice (5/5) fed
406 the chow diet (Fig 3C). In contrast, NR mice (5/5) exhibited a minimal inflammatory
407 response, alongside a slightly increased severity of eosinophilic droplets (average severity
408 3.3 ± 0.3 in R mice vs 4.0 ± 0 in NR mice). Inflammation was characterized by scattered,
409 predominantly periportal foci of lymphocytes, macrophages and neutrophils, associated with
410 minimal bile duct proliferation (Fig 3D, Table 2).
411 After HFHC diet feeding, mice from both groups exhibited minimal to severe macro- and
412 micro-vesicular hepatic lipidosis (Fig 3E-F, Table 2), which progressively increased in
413 severity with time (Table 3). Lipidosis was mainly microvesicular in R mice and occurred with
414 a higher average severity (3.3 ± 0.2) compared to NR mice (1.8 ± 0.2), in which a
415 macrovacuolar pattern was noted more often. Histomorphometrical analysis of lipid droplets
416 in liver sections stained with the ORO method confirmed this result, with livers of R mice
417 showing higher proportions of fat content vs total parenchyma (76.6%) compared to NR
418 mice livers (53.1%) (Fig 3E-F inset). The higher grade of hepatic lipidosis correlated with
419 increased triglycerides levels (1.5 ± 0.1 mg/dL/g vs 2.7 ± 0.1 mg/dL/g) and non-esterified
420 fatty acids (45.5 ± 3.6 mmol/L/g vs 49.9 ± 5.1 mmol/L/g) in livers of R mice vs NR mice (Fig
421 3G-H).
422 Chronic inflammation was also observed in both groups and occurred with higher incidence
423 and severity in NR mice compared to R mice (Fig 4A-L, Table 2), as confirmed by the

quantitative assessment of the CD45 positive reaction (NR mice liver=2121 ± 504.5 CD45+ signal/mm^2 of tissue compared to R mice=889.1 ± 111.9 CD45+ signal/mm^2 of tissue) (Fig 4K-L). In the latter, the inflammatory reaction consisted of scattered aggregates of lymphocytes and macrophages, clustering within or at the periphery of lipidotic areas, associated with minimal bile duct proliferation (Fig 4A and 4C-F).

The liver of NR mice showed, instead, a prominent mixed chronic inflammatory cell infiltration expanding and bridging portal areas, dominated by T and B cells and macrophages, along with fewer plasma cell and neutrophils (Fig 4B and 4G-J). Chronic inflammation was accompanied by minimal fibrosis and prominent bile duct hyperplasia (Fig 4B and 4G-J).

A few (3/30) NR mice exhibited proliferative preneoplastic or neoplastic lesions, such as eosinophilic and clear cell foci of hepatocellular alteration (1/30, Fig 4M) and hepatocellular adenomas (2/30, Fig 4N), which correlated with the nodular masses observed macroscopically. There was no evidence of hepatocellular proliferative lesions in R mice.

Cytoplasmic eosinophilic inclusions occurred with lower severity in mice fed with the HFHC diet, compared to mice given a chow diet. This might be due to aging or to the concurrent high degree of lipidosis, which somehow prevents the formation or detection of these structures. However, similarly to mice fed with the chow diet, eosinophilic inclusions were observed with increased severity in NR mice (2.0 ± 0.3) compared to R mice (0.8 ± 0.2).

No histological abnormality was detected in the remaining examined organs, such as PBAT and aorta, kidney, epididymal WAT, and heart (Fig 4 O-V). In particular, there was no evidence of inflammatory changes. Histomorphometry conducted on renal sections immunostained for CD45 revealed no significant difference between R mice (8610 ± 2175 CD45+/mm^2) and NR mice (8764 ± 2442 CD45+/mm^2) (Fig 4Y).

Thus, we observed two distinct hepatic pathophenotypes under HFHC diet feeding conditions. R mice showed increased lipidosis (mainly microlipidosis) along with mild inflammation, while NR mice showed lower lipidosis levels (mainly macrolipidosis) but higher

incidence and severity of chronic inflammation; along with evidence of hepatocellular proliferative lesions in a small proportion of cases.

Pancreas

In line with the lack of meaningful histological abnormality in the exocrine and endocrine pancreas (data not shown), static glucose stimulation in isolated islets from R mice and NR mice induced a similar increase in insulin secretion in pancreatic islets isolated from R mice and NR mice (Fig 6I).

Cytokines and Transaminase in circulating blood of R mice and NR mice

Plasma cytokines concentrations were similar between R mice and NR mice at time of sacrifice, i.e. after 3 months of HFHC feeding (Fig 5).

However, increased levels of alanine transaminase (ALT) were observed in NR mice after 3 months of HFHC diet compared to chow conditions and compared to R mice at the same time-point (Fig 3J). This confirms liver damage as shown from histology of NR mice.

Lipid metabolism in R mice and NR mice

At the age of 6-7 weeks old, at TNO mice were tested for total cholesterol and triglycerides plasma levels to discriminate between R mice and NR mice. As expected, R mice displayed higher plasma cholesterol (2.9 ± 0.1 mmol/L; i.e.113.2 ± 3.8 mg/dL) compared to NR mice (1.7 ± 0.0 mmol/L; i.e. 68.4 ± 1.9 mg/dL) (Fig 6A). Plasma total cholesterol was measured again upon arrival at our facility on chow diet, and after 1, 2 and 3 months of HFHC diet. Particularly during the first 2 months on HFHC diet, R mice had significantly increased plasma total cholesterol whereas cholesterol levels in NR mice remained similar to the chow-feeding period (Fig 6B). However, after 3 months of HFHC diet, NR mice showed significantly higher values (81.6 ± 3.8 mg/dL) in total cholesterol compared to chow (34.4 ± 2.9 mg/dL) (Fig 6B).

Triglycerides plasma levels were higher in R mice (3.9 ± 0.2 mmol/L; i.e. 341.5 ± 13.7 mg/dL) vs NR mice (0.9 ± 0.0 mmol/L; i.e. 79.4 ± 2.4 mg/mL) at the age of 6-7 weeks old, when measured at TNO (Fig 6C). Similar to total cholesterol, HFHC diet feeding resulted in significantly higher triglycerides concentrations in R mice than in NR mice (Fig 6D). R mice fed the HFHC diet showed uniformly high HDL-cholesterol (HDL-C) level, regardless of the duration of HFHC feeding (Fig 6F).

Free fatty acids ($P<0.05$, Fig 6G) were also significantly increased after 3 months of HFHC diet. No difference was noticed in beta-hydroxybutyrate levels between NR mice and R mice (Fig 6H).

At time of sacrifice, CETP activity, the enzyme that facilitates the transport of cholesterol ester and triglycerides between lipoproteins, was slightly higher in NR mice compared to R mice (4704 ± 324 pmol transferred vs 3488 ± 537 pmol transferred), but the difference was not significant (Fig 6E).

Together, these results demonstrate that the lipid profile in R mice is markedly altered compared to NR mice kept under a HFHC diet feeding regimen.

Bile acid profile in plasma and feces

Plasma levels of bile acids were investigated at time of sacrifice (Fig 7A-L). Overall, R mice showed decreased total bile concentrations; particularly, among free bile acids, cholic acid (CA), deoxycholic acid (DCA), and β-muricholic acid (β-MCA) compared to NR mice (Fig 7A-H). All taurine-conjugated bile acids were also lower in NR mice compared to R mice (Fig 7I-L). Fecal coprostanol excretion was higher in R mice compared to NR mice (Fig 7M). No other differences were found in excreted bile acids between R mice and NR mice (Fig 7M-V).

Discussion

This study aimed to characterize the long-term effect of a 60% kcal fat diet supplemented with 0.25% cholesterol (HFHC) diet on various parameters linked to glucose and lipid metabolism in ApoE3L.CETP mice, a humanized mouse model for the MetS (51). The mutation ApoE3Leiden (14, 60) and the human transgene CETP gene (60) enable these mice' lipid metabolism and profile to be more similar to humans, hence they are one of the suitable animal models available to assess alterations in lipid metabolism in response to HFHC diet (51, 60).

ApoE3L.CETP mice exhibit a dyslipidemic phenotype combined with obesity, insulin resistance and atherosclerosis when administered HFHC diet (7, 20, 23, 54). Interestingly, some degree of phenotypic heterogeneity has been reported among age-matched individuals kept under similar experimental conditions (37). Mice that do not fully display the expected phenotype, i.e., increased body weight and increased triglycerides and total cholesterol levels after HFHC diet exposure, are classified as non-responders (NR) and represent approximately 20% of the total ApoE3L.CETP mice population at TNO (TNO innovation for life, Leiden, The Netherlands; personal communication), the facility that established this mouse model and supplied them for this current study. In fact, NR mice and R mice can already be differentiated at young age when chow fed, based on their prevailing triglyceride and cholesterol plasma levels. To our knowledge, the reasons for these phenotypic differences between R mice and NR mice have never been addressed in detail and it is not clear why ApoE3L.CETP mice sharing the same genetic background exhibit heterogeneous responses to HFHC diet. In addition, in some previous published studies, it is not explicitly mentioned whether NR mice were included (11, 37, 51). This warrants overall caution when evaluating heterogeneous metabolic profiles and responses in ApoE3L.CETP mice that might be related to the dimorphic phenotype of R mice and NR mice (37).

To assess the heterogeneity of the ApoE3L.CETP mice responses and to test the suitability of these mice to study the MetS (37), we investigated in detail body weight gain, feeding

behavior, glucose metabolism, lipid profile, liver histology and inflammation markers over time in several cohorts of mice, and compared mice that were classified as young animals as R mice to mice that were classified as NR mice. In line with what has been previously observed (37), our results confirmed a sharp distinction between R mice and NR mice responses to a HFHC diet. Mice originally classified as R mice were more prone to display the features of the MetS, and exhibited increased body weight, increased subcutaneous and intra-abdominal adipose tissues, increased plasma total cholesterol, triglycerides and HDL-C (11, 37, 51, 60), and reduced glucose sensitivity (31, 63) compared to NR mice. In line with lipid metabolism parameters, histological analysis showed that hepatocellular lipidosis in R mice administered HFHC diet for 2, 3 or 4 months occurred with a high severity progressing over time (average severity: 3.3 ± 0.2, corresponding to 76.6% lipid content in the ORO stained liver sections), whilst NR mice exhibited reduced levels of hepatic lipidosis (average severity: 1.8 ± 0.2, corresponding to 53.1% lipid content in the ORO stained liver sections). Surprisingly, however, NR mice showed prominent hepatic inflammatory and proliferative changes, characterized by severe diffuse chronic lymphohistiocytic infiltration, bile duct proliferation, and, rarely, foci of hepatocellular alteration, and hepatic adenomas. R mice, in contrast, exhibited only minimal inflammation and bile duct proliferation, in the absence of preneoplastic or neoplastic lesions. On an individual basis, there was a clear inverse correlation between the severity of chronic inflammation, and hepatocellular lipidosis, with mice that had high inflammatory scores showing low accumulation of hepatic lipids and decreased body weights, and vice-versa. This indicates that the hepatic pathology seen in NR mice may have a detrimental effect on hepatic function, as indicated by decreased lipid accumulation which we interpret as an inability to metabolize fat properly and increased levels of ALT.

Differences in liver histology between R mice and NR mice were prominent following 2-4 months of HFHC diet feeding, however minimal inflammatory changes were already observed in NR mice at an earlier age (12-16 weeks) while kept on chow diet. Age-matched R mice kept under the same experimental conditions had no histological abnormality in the

559 liver. This suggest that the peculiar NR liver phenotype is likely independent from HFHC,
560 although further studies are needed to confirm this and assess the progression on the
561 disease in NR mice fed a normal chow diet.
562
563 High body weight, high cholesterol and triglycerides levels and a liver histology dominated
564 by lipidosis and minimal inflammatory changes are therefore the salient features of a
565 responsive R phenotype. From our data, it seems that separation between R mice and NR
566 mice conducted at an early stage based on triglycerides levels (<2mmol/L, i.e., 177 mg/mL)
567 seems to be generally predictive of the final phenotype. However, we noted a certain degree
568 of heterogeneity also within R and NR mice groups, leading to a partial overlap between the
569 two groups. There were indeed a few mice within each cohort that did not show the
570 predicted phenotype. For instance, 2/32 in the R mice (6.2%) group might be considered as
571 outliers, i.e. mice originally classified as responders which exhibited low body weight (28.3 g
572 and 35 g), low hepatic lipidosis histological scores (mainly macrolipidosis scored 2 or 3) and
573 high levels of chronic inflammation (scored as 4). In the NR cohort, 3/30 mice (10%)
574 exhibited parameters consistent with a responder mouse, with higher body weight gain (40,
575 49 and 51 g), high lipidosis levels (mainly microlipidosis: scored as 4) and low levels of
576 chronic inflammation (scored 0 or 1).
577 We suggest, therefore, that the introduction of other parameters such as liver histology might
578 be helpful for the final classification of mice. It is important to note that a heterogeneity in the
579 response to HFHC diet is also observed in humans with dysregulated metabolism, and it had
580 to be taken in to account when transferring knowledge from an animal model to humans (28,
581 51).
582 In our study, we maintained the arbitrary classification carried out based only on cholesterol
583 and triglycerides, given that the pathophysiological mechanisms underlying the NR
584 phenotype are unknown and might have a multifactorial origin. Lack of the expected
585 response might be related to the C57BL/6J strain background, as it has been previously
586 shown by Burcelin et al (5) and Hull et al (18). In these studies, it was reported that

C57BL/6J mice do not respond uniformly to HFHC diet feeding. Indeed, some mice gained weight, developed obesity and glucose intolerance, while some others did not (31, 49, 52, 61, 63). The reason for this different response has not been clarified to date.

We originally hypothesized that the difference between R mice and NR mice might be due to different copy numbers of the CETP transgene or to altered transgene expression. While we did not directly measure copy numbers of the CETP transgene in NR mice and R mice, this seems to be unlikely based on our results, as the measured CETP activity did not differ between NR mice and R mice.

It has been previously reported that APOE3Leiden gene overexpressing strains exhibit typical round cytoplasmic eosinophilic droplets, predominantly in the periportal hepatocytes (30). These characteristic inclusions are consistent with aggregates of ApoE3Leiden, however the mechanisms which lead to their accumulation are still poorly understood. We noted that NR mice fed with a chow diet had higher numbers of cytoplasmic inclusions compared to R mice. This difference appeared more evident following HFHC diet feeding. A possible explanation might be that excessive accumulation of ApoE3Leiden protein elicits some form of damage, followed by an inflammatory response; however considerably more work will need to be done to elucidate a possible link between the presence of ApoE3Leiden and a NR phenotype.

Chronic-active inflammation, associated with cholangitis, biliary hyperplasia and proliferative hepatocellular lesions has been described in the liver of mice following chronic infection by *Helicobacter hepaticus* (17, 50). Whilst most animals that carry *Helicobacter spp*. are asymptomatic, susceptible strains and individual mice might manifest clinical signs and lesions and we questioned whether the NR mouse phenotype could be simply the expression of an environmental infection in a proportion of more susceptible mice. FELASA-accredited health check analyses that were conducted at the originating facility, as well as at our premises, revealed that the cohorts of mice employed in the current study did not carry

615 pathogenic etiologic agents. There was no evidence of bacterial organisms in the livers of
616 selected NR mice subjected to a silver stain for the detection of argyrophilic bacteria such as
617 *Helicobacter spp*. Absence of Helicobacter was further confirmed by a qPCR analysis
618 conducted on a pooled liver specimen obtained from R mice and NR mice.
619
620 Since R mice and NR mice did not show any differences in daily food intake or in their
621 feeding behavior, the fact that NR mice did not increase their body weight as much as R
622 mice cannot be explained by a diet-related aspect or a decrease in food intake. Interestingly,
623 energy expenditure also did not differ between R and NR mice but we did not quantify total
624 caloric loss via the feces as a possible mechanism.
625
626 Moreover, glucose metabolism was different between R mice and NR mice, and the
627 difference was more prominent on HFHC diet feeding condition. Particularly, when HFHC
628 diet fed, R mice showed increased basal blood glucose compared to NR mice suggesting
629 that HFHC diet alters the basal glucose metabolism in R mice, and to a lesser extent in NR
630 mice; this is in line with previously published studies (27). We noticed a tendency toward
631 glucose intolerance in R mice similarly to what reported in previous studies conducted with
632 C57BL/6J that also show an increase in basal blood glucose values after HFHC diet (1, 31,
633 49, 52, 62). Even though NR mice showed better glucose tolerance and insulin sensitivity
634 during OGTT and IST, their basal values also slightly increased over time as a potential
635 consequence of HFHC diet and aging (21).
636 We did not find histological abnormalities in the pancreas of R and NR mice that might
637 explain the difference in glucose tolerance. Moreover, insulin secretion after glucose
638 stimulation in islets isolated from R mice and NR mice did not show any differences. This
639 indicates that insulin sensitivity rather than insulin secretion may be more critical for the
640 different glucose tolerance observed in R mice compared to NR mice. We did not perform a
641 hyperinsulinemic euglycemic clamp to investigate the animals' insulin sensitivity in detail.
642

643 Studies in humans (41) and animal models showed the increasing importance of bile acids
644 in modulating body glucose, lipid metabolism and body weight (29). Our findings are
645 contrary from previous data employing the same mouse model (37): surprisingly our results
646 show decreased levels of most of the circulating bile acids (CA, DCA, TDCA, TCDCA,
647 TUDCA, TA/TB-MCA) in R mice compared to NR mice. We hypothesized that these
648 differences in bile acids between R mice and NR mice are due to a dysregulation of bile
649 acids metabolism: in fact, because of the severe liver pathology affecting NR mice, NR mice
650 might be unable to reabsorb and recycle bile acids via hepatic portal vein circulation, hence
651 bile acids might tend to accumulate in the systemic circulation, potentially explaining higher
652 bile acids values in NR mice compared to R mice (6).
653 Indeed, some studies using another mouse model had shown how increased levels of MCA,
654 as seen in our NR mice, might protect against body weight gain, steatosis,
655 hypercholesterolemia and insulin resistance (4). Higher CA levels in NR coupled with lower
656 hepatic TG levels confirmed also previous data (59). Furthermore, different metabolism of
657 bile acids by gut microbiota may also explain why R mice and NR mice might show
658 differences in their bile acids profile (46) but this was not formally tested in our studies.
659
660 Another aspect to take into consideration is that obesity is often accompanied by an
661 increase in the inflammatory status in mice (52) and humans (16, 32, 42). Our data indicate
662 that circulating cytokines did not differ between R mice and NR mice; hence, the
663 inflammation observed in livers of NR mice appears to be organ specific rather than a
664 systemic feature of NR mice compared to R mice (52).
665
666 In summary, our findings suggest that in the context of the heterogeneity previously
667 described for the ApoE3L.CETP mice, two different and distinct phenotypes need to be
668 considered: R mice and NR mice. Particularly, R mice showed the expected phenotype after
669 HFHC diet feeding, confirming its use as a suitable mouse model to describe the MetS
670 especially in relation to lipid metabolism.

With respect to the histological liver finding, these recapitulate partly those seen in NAFLD and likely shares similar pathophysiological mechanisms. As shown by Lau et al (26) several mice models were used to investigate NAFLD as C57BL/6, *db/db* and *ob/ob* mice. Nevertheless, we would rather consider the use of C57BL/6 mice as more accurate model for NAFLD (8, 45), since ApoE3L.CETP mice showed other liver histological features (i.e. minimal bile duct proliferation) that can create confounding factors.

The fact that NR mice do not "respond" to the HFHC diet may therefore indicate that these animals may be protected from the development of diet-induced obesity (and its consequences) in the classical sense, but that the NR mice may in fact be unable to respond adequately to the HFHC diet because of compromised liver function. For this reason, the liver pathology in NR mice would rather not be classified as nonalcoholic steatohepatitis (NASH), i.e. a more aggressive phenotype of NAFLD (36).

We therefore suggest using NR mice with caution; it may be safer to use only R mice in experimental settings and to compare them to appropriate controls within the R cohorts, rather than taking NR mice as control groups. Indeed, we would suggest the use of R mice that are chow fed, huCETP mice or ApoE-/- mice that are fed either chow or the HFHC diet. It may also be advised to reclassify the mice based on the *post mortem* examination and clinical chemistry analysis, to confirm their R mice phenotype based on liver histology and function, respectively.

Limitations and further directions

The current study characterizes in detail the presence of two different phenotypes in ApoE3L.CETP mice, which could explain the heterogeneous metabolic profiles reported previously in this syngenic mouse model. In particular, we provided a detailed analysis of the morphological, metabolic and functional aspects inherent to these two phenotypes and have tried to rule out genetic background influences and environmental factors as a possible reason for the severe inflammatory and proliferative disease observed in the liver of NR

mice. The latter analysis however was limited to an initial investigation and requires further studies to fully understand the pathogenesis leading to the NR mice hepatic phenotype.

In primis, the analysis should be extended also to females and include older animals, to assess its progression over time, and over gender. Particularly we investigated only male because compared to female ApoE3L.CETP they better show the MetS phenotype while females are more suitable for atherosclerosis studies (25, 53). We are aware of the importance of gut microbiota in the scientific community nowadays and further studies with a proper investigation of the gut microbiome of these mice might help in understanding the differences in phenotype between R mice and NR mice.

To better understand the importance of R mice in describing the MetS, we would suggest not only to have other controls as R mice chow fed, huCETP mice, and, also ApoE-/- mice chow fed and HFHC diet. Particularly, only considering proper R mice we would investigate more in details liver function to better explain the mechanisms underneath the MetS**.**

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Table 1: Experimental design

Mice experimental design																								
		TNO facility	Zürich facility																					
			adaptation																					
			chow diet		high fat high cholesterol diet																			
	weeks		-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	mice age	6-7 wks	7-19 wks																					
1st cohort mice	group 1	TC/TG			OGTT				OGTT				OGTT				OGTT							sacrifice/islets
	group 2	TC/TG			IST blood				IST blood				IST blood				IST blood							sacrifice/islets
			8-14 wks																					
2nd cohort mice	group 1	TC/TG			OGTT				OGTT				OGTT				OGTT					sacrifice/islets		
	group 2	TC/TG			IST blood				IST blood				IST blood				IST blood		sacrifice/islets					
	group 3	TC/TG								Indirect calorimetry						sacrifice/histology								

TC: total cholesterol
TG: triglycerides
NEFA: non-esterified fatty acids
BHB: betahydroxybutyrate
OGTT: oral glucose tolerance test
IST: insulin sensitivity test
HDL-C: high.density lipoprotein
ALT: alanine aminotransferase

blood: TC, TG, HDL-C time-point measurements
sacrifice terminal blood collection, NEFA, BHB, cytokines, ALT

727 Table 2: Incidence and severity of histological findings in the liver of R and NR mice
728 administered a chow and HFHC diet

	Chow diet		HFHC diet	
	R	NR	R	NR
Age mice (weeks)	12-16	12-16	28-34	28-34
Liver[a]	5	5	32	30
Hepatocellular Lipidosis[b]	0/5	0/5	32/32	30/30
Grade 0	5	5	0	0
Grade 1	0	0	4	9
Grade 2	0	0	1	15
Grade 3	0	0	8	5
Grade 4	0	0	19	1
Average severity[c]	0 ± 0	0 ± 0	3.3 ± 0.2	1.7 ± 0.2***
Chronic Inflammation[b]	0/5	5/5	14/32	28/30
Grade 0	5	0	18	2
Grade 1	0	5	13	0
Grade 2	0	0	1	3
Grade 3	0	0	0	12
Grade 4	0	0	0	13
Average severity[c]	0 ± 0	1± 0	0.5 ± 0.1	3.2 ± 0.4***
Hepatocellular Eosinophilic Cytoplasmic Inclusions[b]	5/5	5/5	19/32	28/30
Grade 0	0	0	13	2
Grade 1	0	0	12	5
Grade 2	1	0	7	14
Grade 3	3	0	0	9
Grade 4	1	5	0	0
Average severity[c]	3 ± 0.3	4 ± 0	0.8 ± 0.2	2.0 ± 0.3
Focus of the hepatocellular alteration[b]	0/5	0/5	0/32	1/30
Hepatocellular adenomas[b]	0/5	0/5	0/32	2/30

729
730 [a]: number of liver samples analysed, [b]: incidence of the findings, [c]:The average severity of
731 each finding was calculated by summing the severity grades and dividing the total by the
732 number of animals within each group
733
734 ***: $p<0.001$, NR v R, resp., unpaired Student's T test

735 Table 3: Incidence and severity of histological findings in the liver of R and NR mice
736 administered a chow and HFHC diet at different time-points

	Chow diet		HFHC – 8 weeks		HFHC – 12 weeks		HFHC – 16 weeks	
	R	NR	R	NR	R	NR	R	NR
Age mice (wks)	12-16	12-16	20-26	20-26	24-30	24-30	28-34	28-34
Liver [a]	5	5	8	8	11	13	13	9
Hepatocellular lipidosis[b]	0/5	0/5	8/8	8/8	11/11	13/13	13/13	9/9
Grade 0	5	5	0	0	0	0	0	0
Grade 1	0	0	2	4	1	2	1	3
Grade 2	0	0	0	3	1	7	0	5
Grade 3	0	0	2	0	5	4	1	1
Grade 4	0	0	4	1	4	0	11	0
Average severity[c]	0±0	0±0	3.1±0.3	1.7±0.4	3±0.3	2.1±0.2	3.7±0.2	1.8±0.2***
Chronic inflammation[b]	0/5	5/5	4/8	6/8	3/11	13/13	3/13	8/9
Grade 0	5	0	4	2	8	0	10	1
Grade 1	0	5	2	0	3	0	3	0
Grade 2	0	0	1	0	0	2	0	0
Grade 3	0	0	0	1	0	3	0	0
Grade 4			1	5	0	8	0	8
Average severity	0±0	1±0	1±0.5	2.8±0.6*	0.3±0.1	3.5±0.2***	0.2±0.1	3.5±0.4***
Hepatocellular eosinophilic cytoplasmic inclusions[b]	5/5	5/5	5/8	7/8	6/11	13/13	8/13	8/13
Grade 0	0	0	3	1	5	0	5	1
Grade 1	0	0	1	1	5	3	6	1
Grade 2	1	0	4	2	1	7	2	5
Grade 3	3	0	0	4	0	3	0	2
Grade 4	1	5	0	0	0	0	0	0
Average severity	3±0.3	4±0	1.1±0.3	2.1±0.4	0.6±0.2	2±0.2***	0.8±0.2	1.9±0.3**
Focus of the hepatocellular alteration[b]	0/5	0/5	1/8	2/8	1/11	3/13	1/13	4/9
Hepatocellular adenomas[b]	0/5	0/5	0/8	0/8	0/11	1/13	0/13	2/9

737
738 [a]: number of liver samples analysed, [b]: incidence of the findings, [c]: The average severity of
739 each finding was calculated by summing the severity grades and dividing the total by the
740 number of animals within each group
741
742
743 *, **, ***: p< 0.05, 0.01, or 0.001 NR vs R. resp., unpaired Student's T-test

744 **Figures legend**

745 Fig 1: (A) Body weight over a 16-weeks period on HFHC diet in responders (R mice, n=51)
746 and non-responders (NR mice, n=52) ApoE3L.CETP male mice; R mice increased body
747 weight more compared to NR mice with HFHC diet feeding. (B) average 24h food intake and
748 (C) average number of meal bouts in 24h after 8 weeks on HFHC diet in R mice (n=8) and
749 NR mice (n=8); mice displayed no differences between the two groups. (D) Respiratory
750 Exchange Ratio and (E) Energy Expenditure in R mice (n=8) and NR mice (n= 8) mice after
751 10 weeks on HFHC diet; no significant differences were found. (F) Lean and fat mass did not
752 differ between R mice (n=7) and NR mice (n=7). (G) Subcutaneous and intra-abdominal fat
753 mass revealed increased intra-abdominal fat mass in R mice. Data are represented as mean
754 ± SEM. *, *** P< 0.05 or 0.001, resp., NR vs. R, after post hoc Sidak adjustment significant
755 intergroup differences were found by 2-way ANOVA multiple comparison (A, D, E) or
756 unpaired Student's T test, (B, C, F, G).

757

758 Fig 2: Oral glucose tolerance test was performed in responder mice (R mice = 28) and non-
759 responders (NR mice =28) mice on chow (A), and after 1 (B), 2 (C) and 3 (D) months of
760 HFHC diet. R mice from 1 month of HFHC diet displayed glucose intolerance compared to
761 NR mice. (I) Area under the curve (AUC) over 120 min was calculated during the OGTT,
762 above the lowest glucose value of each respective animal.
763 Insulin sensitivity test was performed in responder mice (R mice = 19) and non-responder
764 mice (NR mice = 15) on chow (E), and after 1 (F), 2 (G) and 3 (H) months of HFHC diet. At
765 each time-point R mice displayed lower insulin sensitivity compared to NR mice. (J) Area
766 under the curve (AUC) over 120 min was calculated during IST above the lowest glucose
767 value during the test.
768 Data represented as mean ± SEM., *, **, *** P < 0.05, 0.01, or 0.001 NR vs. R, resp., after
769 unpaired Student's T test (A-D) or after post hoc Sidak adjustment significant intergroup
770 differences were found by 2-way ANOVA multiple comparison (I, J) ~~(E)~~; different letters
771 indicate significant differences.

772 Fig 3: (A - B) microphotographs of livers from R mouse (A) and NR mouse (B) after 3
773 months-HFHC diet. (A) R mouse liver show diffuse light brown discoloration, seems to
774 correlate histologically with mild lipidosis. (B) NR mouse liver show multifocal well
775 demarcated nodular masses (arrows; hepatocellular adenoma). (C and D) HE-stained liver
776 sections from NR and R mice on a chow diet, scale bars = 100 µm: (C) Presence of
777 eosinophilic cytoplasmic droplets (arrowheads), within the hepatocytes of a R mouse, (D)
778 Minimal chronic inflammation (arrow) in a NR mouse liver. (E-F) HE-stained liver sections
779 from mice on a HFHC diet, scale bars = 500 µm. Insets: Oil red O (ORO) stained liver
780 sections from the same mice, scale bars = 20 µm: (E) High grade~~,~~ predominantly
781 microvesicular lipidosis (*) in a R mouse liver. Inset: a high proportion of the hepatocytes
782 contains large cytoplasmic ORO-positive lipid droplets. (F) mild, predominantly
783 macrovesicular lipidosis (*) is observed in the liver of a NR mouse. Inset: ORO-positive lipid
784 content within the hepatocytes is decreased compared to E. (G) triglycerides levels
785 measured in the livers of NR mice (n=10) and R mice (n=10) after sacrifice and (H) non-
786 esterified fatty acids assessed in livers of NR mice (n=17) and R mice (n=15). (I) Absolute
787 liver weight in R and NR mice on 2, 3 and 4 months of HFHC diet. (J) Alanine transaminase
788 (ALT) plasma levels in R mice (n=7) and NR mice (n=9) chow fed and after 4~~3~~ months of
789 HFHC diet. Data are represented as mean ± SEM. *, *** P<0.05 or 0.001, NR vs. R, resp.,
790 after unpaired Student's T test (G-H) or after post hoc Sidak adjustment significant
791 intergroup differences were found by 2-way ANOVA multiple comparison (I and J); different
792 letters indicate significant differences.
793
794 Fig 4: (A - B) HE-stained liver sections from mice fed HFHC diet, scale bars = 100 µm. (A) A
795 R mouse liver with minimal chronic inflammation, characterized by scattered inflammatory
796 cells (open arrowhead) and negligible bile duct proliferation (arrow). Moderate numbers of
797 eosinophilic cytoplasmic droplets (black arrowhead) are detected. (B) A NR mouse liver with
798 a marked chronic inflammatory reaction, represented by mononuclear cell infiltration (black
799 arrowhead) and prominent bile duct proliferation (open arrowhead). (C-J) Liver sections from

a R mouse (C-F) and a NR mouse (55) on a HFHC diet, subjected to immunohistochemistry against the CD3 (T lymphocytes, C/G), CD45R/B220 (B lymphocytes, D-H), F4/80 (macrophages, E-I) and cytokeratin (biliary ductal epithelial cells, F-J) antigens, scale bars = 50 µm. Arrowheads positive reaction. (K-L) Liver sections from a R mouse (K) and a NR mouse (L) on a HFHC diet, subjected to immunohistochemistry against the CD45. CD45-positive cells (black arrowheads) are more numerous in NR mouse liver.

Microphotographs are taken from the same area depicted in A or B, respectively, (M-N) HE-stained liver sections from NR mice on a high fat high cholesterol (HFHC) diet. (M) Focus of cellular alteration (arrowheads), scale bar = 500 µm. Insets: Higher magnification of the lesion, scale bar = 50 µm. (N) Hepatocellular adenoma (arrowheads), scale bar = 1 mm. (O-V) HE-stained aorta and PBAT (O-S), kidney (P-T) epididymal WAT (Q-U), and heart (R-V) from R mouse (O-Q-S-U) and NR mouse (P-R-T-V) scale bars = 100 µm. No histological abnormality is recognized. (W-Y) Quantification of are of CD45-positive stained cells/mm2 in the liver (W) and kidneys (Y) of NR mice and R mice. Data are represented as mean ± SEM. *, $P<0.05$, NR vs. R, resp., after unpaired Student's T test.

Fig 5: Plasma cytokines in plasma from non-responder (NR mice, n=7) and responder (R mice, n=9) mice at the time of sacrifice: (A) Interferon- γ, IFN-γ. (B) Interleukin 10, IL-10. (C) Interleukin 12p70, IL-12p70. (D) Interleukin 1β, IL-1β. (E) Interleukin 2, IL-2. (F) Interleukin 4, IL-4. (G) Interleukin 5, IL-5. (H) Interleukin 6, IL-6. (I) Keratinocyte chemoattractant/growth-regulated oncogene, KC-PRO and (J) tumour necrosis factor α, TNF-α. No difference between the two groups was found. Data are represented as mean ± SEM. *, $P< 0.05$, NR vs. R, resp after unpaired Student's T test.

Fig 6: Plasma lipid profile in non-responder (NR mice, n=11) and responder (R mice, n=13) mice at different time points (A-H). Total cholesterol (A, B) and triglycerides (C, D) at aged 6-7 weeks, analysis performed from TNO when discrimination between R mice and NR mice

827 took place (A, C) and at time 0 (chow fed upon arrival in our facility), and after 1, 2 and 3
828 months of HFHC diet (B, D). (E) CETP activity at time of sacrifice in NR mice (n=10) and R
829 mice (n=8); no difference was found. (F) HDL cholesterol (HDL-C) on chow diet upon arrival,
830 and after 1, 2 and 3 months of HFHC. (G) non-esterified fatty acids (NEFA) were higher in R
831 mice vs NR mice, and (H) and beta-hydroxybutyrate (BHB) in NR mice (n=10) and R mice
832 (n=10). (I) Insulin secretion expressed as a percentage of total insulin content after glucose
833 stimulated insulin secretion test (GSIS) in islets isolated from NR mice and R mice and
834 cultured for 4 days. Data are represented as mean ± SEM. *, *** P< 0.05 or 0.001, resp., NR
835 vs. R, after post hoc Tukey adjustment significant intergroup differences were found by 2-
836 way ANOVA multiple comparison (B, D, F, I) or unpaired Student's T test, (A, C, E, G, H).
837 Different letters indicate significant differences
838
839 Fig 7: Total plasma bile (A-L) and total coprostanol (M), cholesterol (N) and total bile acid
840 (O-U) in NR mice and R mice feces at time of sacrifice. (A) Total bile acids, total BA (n=17,
841 20). (B) cholic acid, CA (n=14, 15). (C) chenodeoxycholic acid, CDCA (n=14, 10). (D)
842 deoxycholic acid, DCA (n=17, 15). (E) α-muricholic acid, α -MCA (n=13, 6). (F) β-muricholic
843 acid, β-MCA (n=15, 14). (G) tauro cholic acids, TCA levels (n=15, 15). (H) taurodeoxycholic
844 acid, TDCA (n=17, 15). (I) taurocheno deoxycholic acid, TCDCA (n=16,10), (J)
845 tauroursodeoxycholic acid, TUDCA (n=17, 20), (K) tauro-α-muricholic acid, Tα-MCA (n=16,
846 14) and (L) tauro-β-muricholic acid, Tβ-MCA (n=17, 12). (M) Coprostanol, Copr (n=5, 2). (N)
847 cholesterol, Chol (n=6, 5). (O) total bile acids, total BA (n= 6, 5). (P) dihydrocholestero,l
848 diHyChol (n=5, 5). (Q) cholic acid, CA (n=6, 5). (R) allo cholic acid, alloCA (n=6, 5). (S)
849 deoxy cholic acid, DCA (n=6, 5). (T) α-muricholic acid, α -MCA (n=6, 5). (U) β-muricholic
850 acid, β-MCA (n= 6, 5). (V) ω-muricholic acid, ω-MCA (n= 6, 5). (J). Data are represented as
851 mean ± SEM. *, **, *** P< 0.05, 0.01 or 0.001, resp., NR vs. R, using unpaired Student's T
852 test.

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